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By /Jo Ann Honcik Dallara/
Jo Ann Honcik Dallara

PATENT
Attorney Docket No.: 016866-003810US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

YIP et al.

Application No.: 10/088,970

Filed: July 19, 2002

For: PROSTATE CANCER MARKER PROTEINS

Customer No.: 53671

Confirmation No. 6649

Examiner: Fetterolf, Brandon J

Technology Center/Art Unit: 1642

RULE 132 DECLARATION BY DR. TAI-TUNG YIP

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, **Dr. Tai-Tung Yip**, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true. The Exhibits (1 and 2) attached hereto are incorporated herein by reference.

2. I received a Ph.D. in Biochemistry from the Chinese University of Hong Kong, Faculty of Science in 1985.

3. I am presently employed by Ciphergen as a Senior Research Fellow and I am primarily responsible for clinical and basic research in SELDI-TOF-MS.

4. I have read and am familiar with the contents of the subject patent application. I understand that the Examiner has a rejection of the pending claims based on enablement. The Examiner is concerned that practice of the invention, as previously claimed, would require undue experimentation to fully practice. More specifically, the Examiner was concerned about the reproducibility of our results. The primary concerns were over whether the surface chemistry of the MS probe would affect our results, whether the work was reproducible across a large patient population and whether we could distinguish between benign prostate hyperplasia and prostate cancer from sample sources beyond prostate serum (seminal fluid). Secondary concerns were whether sample handling, statistical analyses and patient conditions would affect our results.

Below I address the primary concerns individually. The secondary concerns are addressed by a single response.

5. IS THE PHENOMENON OF AN ABUNDANCE OF LOWER WEIGHT PROTEINS IN PROSTATE CANCER SAMPLES DETECTABLE WITH OTHER MS PROBE SURFACES?

The answer is yes. We looked at three different surfaces to determine the answer to this very question. Attached to this declaration as Exhibit 1 are copies of MS profiles done using two other chip surfaces. Figure 6 of our patent application illustrates the results with an SCX1 surface which is described in our specification at page 33, lines 26-31 and uses a sulfonated polystyrene as a capture agent. Exhibit 1 describes the similar serum samples studied on a Ciphergen IMAC Ni(II) chip and our H4 chip. The IMAC chip is described in detail on page

31, lines 16-23. Our H4 is a good general protein capture surface that mimics reversed phase chromatography with C16 functionality. H4 is described in our specification at page 32, lines 15-32.

From Exhibit one, it should be clear that the generation of low molecular weight peptide products is observed in our mass spectroscopic instruments using three different capture surfaces. Obviously, the surface chemistry needs to have the capacity to capture an appropriate range of proteins.

6. IS THE PHENOMENON OF AN ABUNDANCE OF LOWER WEIGHT PROTEINS IN PROSTATE CANCER SAMPLES DETECTABLE IN OTHER PATIENTS?

The answer is yes. I have attached two papers reporting on work by my co-inventors using Ciphergen mass spectroscopy equipment that looked at this very question. The Adam *et al.* study (Exhibit 2) used samples from hundreds of patient serum samples including control patients, patients with BPH and those with two types of prostate cancer. While the work reported on MS protein fingerprinting coupled with a sophisticated pattern matching algorithm, the results accurately reflect our earlier work. The authors wrote on page 3609, 2nd col:

> Using a standardized test set, we demonstrate proof of principle that our SELDI protein profiling approach can accurately discriminate PCA from patients with BPH and men of the same age who do not have prostate disease.

The authors also write on page:

> The successful use of the prostate classification system described herein relies entirely on the protein fingerprinting of the nine masses. Because these masses were found to be reproducibly reliably detected, only the mass values are required to make a correct classification or diagnosis. Knowing the identities for the purpose of differential diagnosis is not required.

Finally, evidence of the low molecular weight peptide products as prostate cancer markers can be clearly seen in Figure 2 (c) on page 3611.

Cazares *et al.* (Exhibit 3) provides similar evidence of successful application of our invention using prostate tissues samples from 9 patients. See the results section where the authors (including co-inventor George Wright) wrote in the abstract on page 2541:

> *Results:* Several small molecular mass peptides or proteins (3000-5000 Da) were found in greater abundance in PIN and PCA cell lysates.

7. IS THE PHENOMENA OF AN ABUNDANCE OF LOWER WEIGHT PROTEINS IN PROSTATE CANCER PATIENTS DETECTABLE IN SAMPLES OTHER THAN SERUM?

Exhibits 2 and 3 provide evidence that the invention is applicable to samples other than from seminal fluid. Exhibit 2 shows data from blood serum and Exhibit 3 provides data from prostate tissue biopsies.

8. THE REMAINING SECONDARY CONCERNS ARE ISSUES THAT ARE ROUTINELY ADDRESSED BY COMPETENT LABORATORY TECHNICIANS AND DO NOT INVOLVE UNDUE EXPERIMENTATION.

The Examiner takes note of several papers raising concerns about the use of SELI-TOF-MS for diagnostic purposes. These include Grizzle *et al.* which includes co-inventor George Wright's colleague, John Semmes as co-author and by Diamandis. None of the papers challenges the use of MS to accurately detect proteins and for the results to reflect differences in disease states. Grizzle mentions issues with instrumentation drift that might make routine clinical diagnosis more complicated than if measured by individual peaks, a problem this invention avoids. Grizzle also mentions that patterns of eating, age and familial relationships may influence the results. In addition, site collection and storage of samples including the containers are important considerations. Grizzle also mentions that large and abundant proteins

such as albumin can also bind small proteins and that the removal of albumin might cause under representation of the low molecular markers. Diamandis make comments of similar character.

In response, applicants fully acknowledge these issues as relevant ones. It is hoped that the patient samples described by Adams *et al.* and Cazares *et al.* addresses most of these concerns. However, the fact that a cheap cup might leach plastic or vinyl components and render the samples useless or that sample handling must be uniform and be in a stable environment to avoid sample degradation, are issues for any assay. But, I submit that these issues are not of the type that give rise to enablement concerns for patent claims. They are obvious problems to avoid or to solve if one simply follows the preferred methods described in the specification. Thus the stated problems should not be of serious concern in that they are not problems requiring undue experimentation to avoid.

This Declarant has nothing further to say.

Dated: June 22, 2006

Tai-Tung Yip, Ph.D

attachments: Exhibit 1 [MS Spectra]
Exhibit 2 [Adam, *et al.* 2002]

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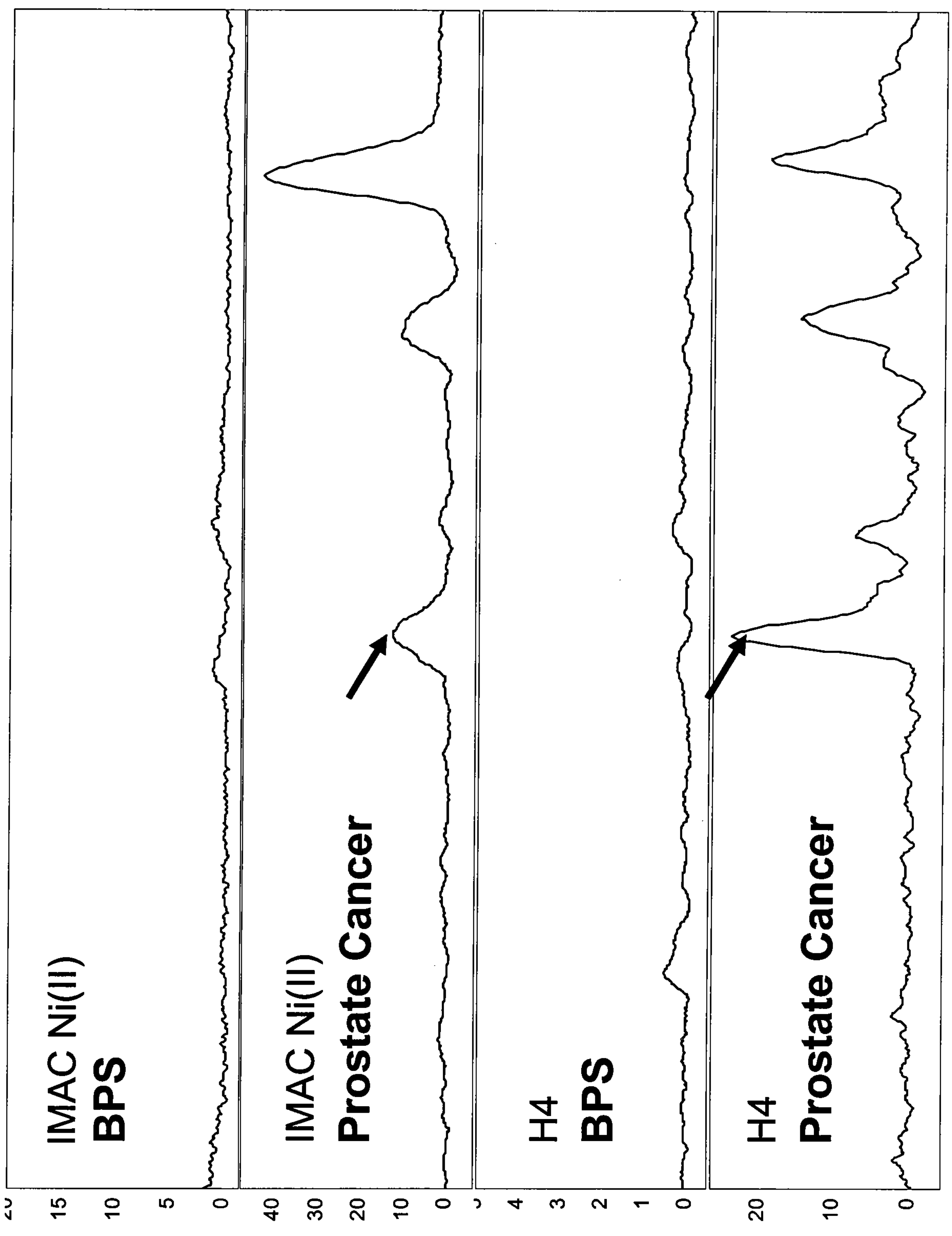
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EXHIBIT 1

[CANCER RESEARCH 62, 3609–3614, July 1, 2002]

Advances in Brief

Serum Protein Fingerprinting Coupled with a Pattern-matching Algorithm Distinguishes Prostate Cancer from Benign Prostate Hyperplasia and Healthy Men[1]

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Abstract

The prostate-specific antigen test has been a major factor in increasing awareness and better patient management of prostate cancer (PCA), but its lack of specificity limits its use in diagnosis and makes for poor early detection of PCA. The objective of our studies is to identify better biomarkers for early detection of PCA using protein profiling technologies that can simultaneously resolve and analyze multiple proteins. Evaluating multiple proteins will be essential to establishing signature proteomic patterns that distinguish cancer from noncancer as well as identify all genetic subtypes of the cancer and their biological activity. In this study, we used a protein biochip surface enhanced laser desorption/ionization mass spectrometry approach coupled with an artificial intelligence learning algorithm to differentiate PCA from noncancer cohorts. Surface enhanced laser desorption/ionization mass spectrometry protein profiles of serum from 167 PCA patients, 77 patients with benign prostate hyperplasia, and 82 age-matched unaffected healthy men were used to train and develop a decision tree classification algorithm that used a nine-protein mass pattern that correctly classified 96% of the samples. A blinded test set, separated from the training set by a stratified random sampling before the analysis, was used to determine the sensitivity and specificity of the classification system. A sensitivity of 83%, a specificity of 97%, and a positive predictive value of 96% for the study population and 91% for the general population were obtained when comparing the PCA *versus* noncancer (benign prostate hyperplasia/healthy men) groups. This high-throughput proteomic classification system will provide a highly accurate and innovative approach for the early detection/diagnosis of PCA.

Introduction

The number of PCA[4] cases has tripled during the past decade due to the widespread use of serum PSA testing and DRE (1). Although these efforts have allowed for increased identification of individuals with cancer, overall "early" detection or determination of aggressive cancers is needed. PSA is currently the best overall serum marker for PCA in clinical use. Nevertheless, the PSA test lacks specificity (2, 3), limiting its use as an early detection biomarker, and its relation to biological activity has been questioned (4). It is important that additional diagnostic biomarkers be identified to reduce PCA mortality. However, because of the robust molecular and cellular heterogeneity of PCA, it is likely that a combination or a panel of biomarkers will be required to improve the early detection of PCA.

The study of the cell's proteome presents a new horizon for biomarker discovery. Two-dimensional PAGE has been the classical approach to explore the proteome for separation and detection of differences in protein expression (5, 6). Advances in two-dimensional gel electrophoresis technology coupled with robotics and software programs for identifying potential protein alterations have improved this proteomic system. Nevertheless, two-dimensional gel electrophoresis is still cumbersome, labor intensive, suffers reproducibility problems, and is not readily transformed into a clinical assay. Advances have also been made in mass spectrometry to achieve high-throughput separation and analysis of proteins (7–9). One of the recent advances is the ProteinChip system manufactured by Ciphergen Biosystems, Inc. (Fremont, CA). This system uses SELDI time-of-flight mass spectrometry to detect proteins affinity-bound to a protein chip array (10, 11). This system is a novel, extremely sensitive, and rapid method to analyze complex mixtures of proteins and peptides. Initial studies from our laboratory established the potential of SELDI for discovery and profiling of prostate and bladder cancer biomarkers in body fluids and cell lysates (12, 13).

The objective of this study was to determine whether SELDI protein profiling of serum coupled with an artificial intelligence data analysis algorithm could effectively differentiate PCA from BPH and unaffected HM. Using a standardized test set, we demonstrate proof of principle that our SELDI protein profiling approach can accurately discriminate PCA from patients with BPH and men of the same age who do not have prostate disease. Our results form the basis for initiating further evaluation and validation to assess the potential of this SELDI proteomic classification system for the early detection and diagnosis of PCA, and further study is warranted to establish profiles that identify the clinically important lethal cancers.

Materials and Methods

Serum Samples. Serum samples were obtained from the Virginia Prostate Center Tissue and Body Fluid Bank. The serum procurement, data management, and blood collection protocols were approved by the Eastern Virginia Medical School Institutional Review Board. Blood samples from patients diagnosed with either PCA or BPH were procured from the Department of Urology, Eastern Virginia Medical School, and the HM cohort was obtained from free screening clinics open to the general public. Only pretreatment samples obtained at the time of diagnosis of PCA or BPH were used for this study. After obtaining informed consent from the patient, the sample was

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[1] Supported by the National Cancer Institute Early Detection Research Network (http://edrn.nci.nih.gov; Grant CA85067) and the Virginia Prostate Center (http://www.evms.edu/vpc/).

[2] B-L. A., Y. Q., and G. L. W. contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, 700 West Olney Road, Norfolk, VA 23501. Phone: (757) 446-5662; Fax: (757) 624-2255; E-mail: wrightgl@evms.edu.

[4] The abbreviations used are: PCA, prostate cancer; PSA, prostate-specific antigen; SELDI, surface enhanced laser desorption/ionization mass spectrometry; BPH, benign prostate hyperplasia; HM, healthy men; EAM, energy-adsorbing molecule; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; DI, deionized. DRE, digital rectal examination.

EXHIBIT 2

collected into a 10-cc Serum Separator Vacutainer Tube and centrifuged 30 min later at 375,000 rpm for 5 min. The serum was distributed into 500-μl aliquots and stored frozen at -80°C. A quality control sample was prepared by pooling an equal amount of serum from each specimen of the age-matched HM group and storing 100-μl aliquots at -80°C. The quality control serum sample was used to determine reproducibility and as a control protein profile for each SELDI experiment.

Patient and Donor Cohorts. Specimens from four groups of patients were used in this study: (*a*) 97 age-matched HM (control); (*b*) 92 patients with BPH; (*c*) 99 patients diagnosed with organ-confined PCA (T_1/T_2); and (*d*) 98 patients diagnosed with non-organ-confined PCA (T_3/T_4). A donor was selected for the HM group if he had a normal DRE, a PSA < 4.0 ng/ml, and no evidence of prostatic disease. The HM group consisted of 48 Caucasian and 48 African-American males ranging in age from 51–70 years (mean age, 60 years). There were 33 Caucasians, 2 African Americans, and 57 men of unknown race in the BPH patient group, ranging in age from 48–86 years (mean age, 67 years). The BPH patients were selected if they had PSA values between 4 and 10 ng/ml, low PSA velocities (*i.e.*, PSA velocity <0.7 ng/ml/year), and multiple negative biopsies. The number of biopsies was two (73 cases), three (13 cases), and four (6 cases). The organ-confined PCA group (T_1/T_2) consisted of 76 Caucasians, 20 African Americans, 1 Asian, and 2 men of unknown race with ages ranging from 50–89 years (mean age, 71 years). For the non-organ-confined PCA group (T_3/T_4), there were 80 Caucasians, 16 African Americans, and 2 men of unknown race, ranging in age from 44–87 years (mean age, 69 years). The range and mean PSA values for the groups were as follows: a 0.15–3.83 ng/ml (1.32 ng/ml) for the HM group [86 members of this group had a PSA < 2.5 ng/ml (the latter were considered to be a low-risk group)]; (*b*) 0.0–10.91 ng/ml (4.60 ng/ml) for the BPH group; (*c*) 0.0–95.16 ng/ml (10.10 ng/ml) for the organ-confined PCA (T_1/T_2) group; and (*d*) 0.0–8752 ng/ml (206.93 ng/ml) for the non-organ-confined PCA (T_3/T_4) group.

SELDI Protein Profiling. Various chip chemistries (hydrophobic, ionic, cationic, and metal binding) were initially evaluated to determine which affinity chemistry provided the best serum profiles in terms of number and resolution of proteins. The IMAC-Cu metal binding chip was observed to give the best results. IMAC-3 chips (Ciphergen Biosystems, Inc.) were coated with 20 μl of 100 mM $CuSO_4$ on each array, placed on a TOMY Micro Tube Mixer (MT-360; Tomy Seiko Co., Ltd.), and agitated for 5 min. The chips were rinsed 10 times with DI water, and 20 μl of 100 mM sodium acetate were added to each array and shaken for 5 min to remove the unbound copper. The chips were rinsed again with DI water (10 times) and put into a bioprocessor (Ciphergen Biosystems, Inc.), which is a device that holds 12 chips and allows application of larger volumes of serum to each chip array. The bioprocessor was washed and shaken on a platform shaker at a speed of 250 rpm for 5 min with 200 μl of PBS in each well. This was repeated twice more, and each time the PBS buffer was discarded by inverting the bioprocessor on a paper towel. Serum samples for SELDI analysis were prepared by vortexing 20 μl of serum with 30 μl of 8 M urea/1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid in PBS in a 1.5-ml microfuge tube at 4°C for 10 min. One hundred μl of 1 M urea with 0.125% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid were added to the serum/urea mixture and vortexed briefly. PBS was added to make a 1:5 dilution and placed on ice until applied to a protein chip array. Fifty μl of the diluted serum/urea mixture were applied to each well, and the bioprocessor was sealed and shaken on a platform shaker at a speed of 250 rpm for 30 min. The serum/urea mixture was discarded, and the PBS washing step was repeated three times. The chips were removed from the bioprocessor, washed 10 times with DI water, air dried, and stored in the dark at room temperature until subjected to SELDI analysis. Before SELDI analysis, 0.5 μl of a saturated solution of the EAM sinapinic acid in 50% (v/v) acetonitrile, 0.5% trifluoroacetic acid was applied onto each chip array twice, letting the array surface air dry between each sinapinic acid application. Chips were placed in the Protein Biological System II mass spectrometer reader (Ciphergen Biosystems, Inc.), and time-of-flight spectra were generated by averaging 192 laser shots collected in the positive mode at laser intensity 220, detector sensitivity 7, and a focus lag time of 900 ns. Mass accuracy was calibrated externally using the All-in-1 peptide molecular mass standard (Ciphergen Biosystems, Inc.).

Data Analysis. The data analysis process used in this study involved three stages: (*a*) peak detection and alignment; (*b*) selection of peaks with the highest discriminatory power; and (*c*) data analysis using a decision tree algorithm. A stratified random sampling with four strata [PCA (T_1/T_2), PCA (T_3/T_4), BPH, and HM] was used to separate the entire data set into training and test data sets before the analysis. The training data set consisted of SELDI spectra from 167 PCA, 77 BPH, and 82 normal serum samples. The validity and accuracy of the classification algorithm were then challenged with a blinded test data set consisting of 30 PCA, 15 BPH, and 15 normal samples.

Peak Detection. Peak detection was performed using Ciphergen SELDI software versions 3.0 β and 3.0.[5] The mass range from 2,000–40,000 Da was selected for analysis because this range contained the majority of the resolved protein/peptides. The molecular masses from 0–2,000 Da were eliminated from analysis because this area contains adducts and artifacts of the EAM and possibly other chemical contaminants. Peak detection involved (*a*) baseline subtraction, (*b*) mass accuracy calibration, and (*c*) automatic peak detection. The software program calculates noise, peak area, and filter based on the criteria selected by the operator for data analysis. The settings used for this study were as follows: (*a*) fitting window width = 100 data points; (*b*) average noise = 10 points; (*c*) peak area calculated using the slope-based method; (*d*) low minimum valley depth = 10 times noise; (*e*) high minimum valley depth = 0.5 times noise; (*f*) low and high sensitivity of peak height = 10 and 2 times noise, respectively; (*g*) auto peak detection slider = 8 for mass range 2–4 kDa, 11 for mass range 4–8 kDa, and 8 for mass range 8–40 kDa. An average of 81 peaks was detected in each spectrum.

Peak Alignment. All of the labeled peaks from 772 spectra were exported from SELDI to an Excel spreadsheet. A PeakMiner algorithm,[6] developed in-house, was used to align peaks and perform statistical analysis. Peaks were first sorted by mass, and a mass error value was calculated for each peak. The mass error score, the measurement of mass difference between peak X and peak $X + 1$, is calculated for each peak using $(Mpx - Mpx + 1)/Mpx$, where Mpx is the mass value of peak X. For example, if the mass error score was $<0.18\%$, peak X and peak $X + 1$ would align into one peak, representing the same protein in each sample. If the mass error was $>0.18\%$, then peak X and peak $X + 1$, would be considered two distinct peaks. This is an iterative process throughout all of the labeled peaks.

Feature Selection. The power of each peak in discriminating normal *versus* PCA, normal *versus* BPH, and BPH *versus* PCA was determined by estimating the AUC, which ranges from 0.5 (no discriminating power) to 1.0 (complete separation).

Decision Tree Classification. Construction of the decision tree classification algorithm was performed as described by Breiman *et al.* (14) with modifications,[7] using a training data set consisting of 326 samples (82 normal, 77 BPH, and 167 PCA samples). Classification trees split up a data set into two bins or nodes, using one rule at a time in the form of a question. The splitting decision is defined by presence or absence and the intensity levels of one peak. For example, the answer to "Does mass A have an intensity less than or equal to X" splits the data set into two nodes, a left node for yes and a right node for no. This splitting process continues until terminal nodes or leaves are produced or further splitting has no gain. Classification of terminal nodes is determined by the group ("class") of samples (*i.e.*, PCA, BPH, or HM) representing the majority of samples in that node. A "cost" function is calculated that reflects the heterogeneity of each node: $-\log L = -\Sigma nj \log(pj)$ where L is the likelihood of the multinomial distribution, nj is the number of samples in class j, and pj is the probability of class j. Peaks selected by this process to form the splitting rules are the ones that achieve the maximum reduction of cost in the two descendant nodes.

Statistical Analyses. The AUC was computed to identify the peaks with the highest potential to discriminate the three groups, based on the probability that the test result from a diseased individual is more indicative of disease than that from a nondiseased individual (15). A Bayesian approach was used to calculate the expected probabilities of each class in each terminal node (16), and their 95% confidence intervals were calculated using the posterior Dirichlet distribution (16). The 95% confidence intervals were calculated by generating and sorting 4000 samples for the posterior Dirichlet distribution, and the 100[th] and 3900[th] sample were considered as the lower and upper bounds of the 95% confidence intervals, respectively. Specificity was calculated as the ratio of the number of nondisease samples correctly classified to

[5] Internet address: www.chiphergen.com.
[6] Internet address: www.evms.edu/vpc/seldi.
[7] Internet address: http://140.107.129.65/stat_methods.htm.

the total number of nondisease samples. Sensitivity was calculated at the ratio of the number of correctly classified diseased samples to the total number of diseased samples. The PPV for the study population was calculated by dividing the number of true PCA positives by the sum of the number of true PCA positives plus the number of false PCA positives. The NPV for the study population was calculated by dividing the number of true negative nondisease samples (BPH/HM) by the sum of the number of false negative plus the number of true negative nondisease samples (BPH/HM). The PPV and NPV for PCA *versus* noncancer (BPH/HM) in the general population were calculated as follows: PPV (for population) = sensitivity * rho/[sensitivity * rho + (1 − specificity) * (1 − rho)]; and NPV (for population) = specificity * (1 − rho/[specificity) * (1 − rho) + (1 − sensitivity) * rho], where rho is prevalence in the population.

Results

Data Analysis. Peak detection using the SELDI software program detected 63,157 peaks in the 2–40-kDa mass range after analysis of 772 spectra (386 spectra in duplicate, with approximately 81 peaks/spectrum). Of these, 779 peaks were identified after the clustering and peak alignment process. The AUC was calculated for each of the 779 peaks. No single peak was identified that had an AUC of 1.0, indicating that there was not a peak detected that alone could completely separate two groups (*i.e.*, HM *versus* PCA, HM *versus* BPH, or BPH *versus* PCA) or three groups (PCA *versus* BPH *versus* HM). Of the 779 peaks, 124 had an AUC ≥ 0.62. Those with an AUC < 0.62 were considered irrelevant for classification. These 124 peaks identified in the training set were then used to construct the decision tree classification algorithm. Fig. 1 is a flow diagram that summarizes the process from peak detection to sample classification. The classification algorithm used nine masses between 4 and 10 kDa (4475, 5074, 5382, 7024, 7820, 8141, 9149, 9507, and 9656 Da) to generate 10 terminal nodes (L1–L10; Fig. 2*A*). Once the algorithm identifies the most discriminatory peaks, the classification rule is quite simple. For example, if an unknown sample has no peak at mass 7819.75 ("root" node) but has a peak at mass 7024.02, then the sample is placed in terminal node L1 and classified as PCA. If the sample is placed in L2, it will be assigned to BPH. Another example of this splitting process is shown in Fig. 2*B*, in which four masses between 5 and 10 kDa are used to assign 46 of the 167 PCA samples to terminal node L7. Based

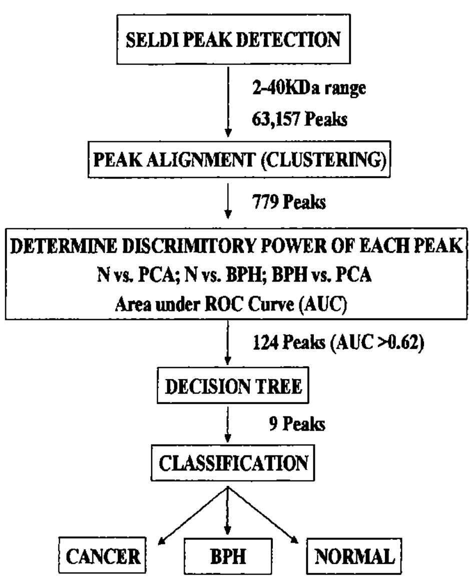

Fig. 1. Flow diagram showing the processes involved in development of the classification tree analyses program. *N* and *Normal*, unaffected HM.

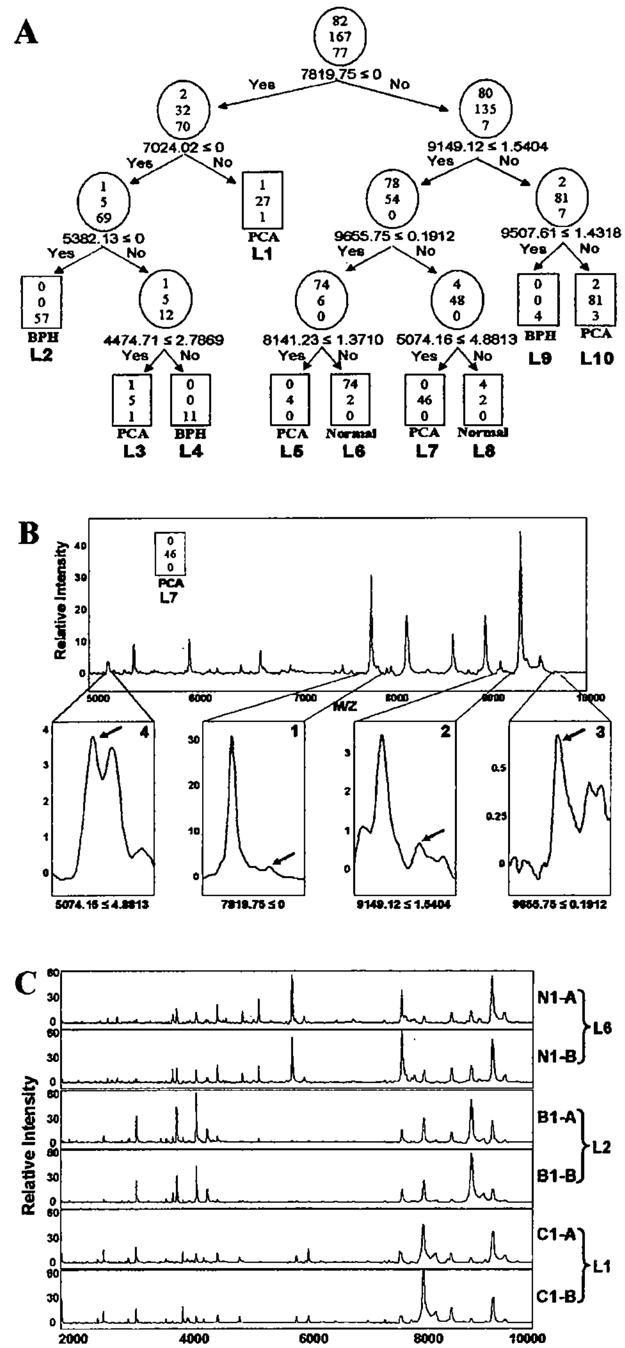

Fig. 2. Classification of the prostate disease and nondisease samples in the training data set. *A*, diagram of decision tree analyses. The root node (*top*) and descendant nodes are shown as *ovals*, and the terminal nodes (*L1–L10*) are shown as *rectangles*. The numbers in each node represent the classes [*top number*, number of HM (normal control) samples; *middle number*, number of PCA samples; *bottom number*, number of BPH samples]. The first number under the root and descendant nodes is the mass value followed by the peak intensity value. For example, the mass value under the root node is 7819.75 kDa, and the intensity is ≤0. *B*, representative example of a SELDI spectrum showing the combination of four peak masses required to correctly classify the sample as PCA in the L7 terminal node. The *arrows* in the magnified panels identify the protein peaks used in the classifier, and the numbers *1–4* in the *top right corner* indicate the order the decision tree takes in assigning the sample to the L7 terminal node. The *first number* under each panel is the mass, and the *second number* is the peak intensity. *C*, example of the reproducibility of the SELDI and decision tree classification analyses. Serum samples randomly selected and repeated 18 months (*B*) after the initial SELDI analysis (*A*) showed similar spectra and were correctly classified to the appropriate terminal node by the decision tree algorithm; in this example either terminal node L1, L2, or L6. *N1*, sample from a healthy male donor; *B1*, sample from a patient with BPH; *C1*, sample from a patient with PCA.

Table 1 *Expected probabilities and the 95% confidence levels for each of the classes assigned to the 10 terminal nodes*

Node	Class	Observation	Probability	95% Confidence level (low, high)
L1	HM	1	0.0625	0.0081, 0.1693
	PCA	27	0.8750	0.7423, 0.9630
	BPH	1	0.0625	0.0087, 0.1698
L2	HM	0	0.0167	0.0005, 0.0584
	PCA	0	0.0167	0.0004, 0.0628
	BPH	57	0.9667	0.9072, 0.9952
L3	HM	1	0.2000	0.0247, 0.4793
	PCA	5	0.6000	0.3027, 0.8592
	BPH	1	0.2000	0.0248, 0.4753
L4	HM	0	0.0714	0.0018, 0.2509
	PCA	0	0.0714	0.0019, 0.2579
	BPH	11	0.8571	0.6311, 0.9823
L5	HM	0	0.1429	0.0040, 0.4725
	PCA	4	0.7143	0.3557, 0.9567
	BPH	0	0.1429	0.0040, 0.4504
L6	HM	74	0.9494	0.8950, 0.9858
	PCA	2	0.0380	0.0082, 0.0879
	BPH	0	0.0127	0.0003, 0.0459
L7	HM	0	0.0204	0.0005, 0.0738
	PCA	46	0.9592	0.8893, 0.9951
	BPH	0	0.0204	0.0005, 0.0726
L8	HM	4	0.5556	0.2458, 0.8416
	PCA	2	0.3333	0.0836, 0.6544
	BPH	0	0.1111	0.0032, 0.3732
L9	HM	0	0.1429	0.0034, 0.4560
	PCA	0	0.1429	0.0037, 0.4830
	BPH	4	0.7143	0.3354, 0.9566
L10	HM	2	0.0337	0.0068, 0.0784
	PCA	81	0.9213	0.8595, 0.9674
	BPH	3	0.0449	0.0123, 0.0964

on the stochastic nature of reality, misclassification of a new sample cannot be ruled out even for a pure node that contains only one sample type, for example, L2, which contains only BPH samples. To obtain an idea of whether an unknown sample would be correctly classified or misclassified, the expected probability and 95% confidence level was calculated for each class in the 10 terminal nodes (Table 1). The expected probabilities for HM and PCA samples to be misclassified in L2, for example, are 1.67%. Although not zero, the likelihood of HM or PCA samples being assigned to this node is extremely low; whereas BPH has a 96.67% chance of being correctly classified to L2 (with the 95% confidence interval between 90.72% and 99.52%). The probability of incorrect assignment of samples increases in nodes that contain few majority samples or when only a few samples are assigned to the node, as, for example, terminal nodes L3, L5, and L9 (Fig. 2*A*).

A summation of the classification results from the 10 terminal nodes is presented for the training and test sets in Table 2. The classification algorithm correctly predicted 93.51–97.59% of the samples for each of the three groups in the training set (Table 2A), for an overall correct classification of 96%. The algorithm correctly predicted 90% (54 of 60) of the test samples, with all 15 samples from HM, 93% (14 of 15) of the BPH samples, and 83% (25 of 30) of the PCA samples being correctly classified (Table 2B). Three of the misclassified HM cases in the training set had PSA values < 2.5 ng/ml (*i.e.*, 0.15, 0.76, and 1.52 ng/ml), considered a low-risk group, and the fourth case had a PSA of 3.02 ng/ml (*i.e.*, high-risk group). Therefore, no correlation for the misclassification of four of the HM cases with PSA levels could be made.

The sensitivity and specificity of the classification system for differentiation of disease from the nondisease groups are presented in Table 2C. When comparing PCA *versus* noncancer (BPH/HM), the sensitivity was 83% (25 of 30), and the specificity was 97% (29 of 30). A sensitivity of 83% was also obtained when comparing PCA *versus* HM (25 of 30) or PCA *versus* BPH (25 of 30), whereas the specificity was 100% (15 of 15) for PCA *versus* HM and 93% (14 of 15) for PCA *versus* BPH. The PPV and NPV for the study population were 96.15% and 96.67%, respectively. When considering an estimated 30% prevalence of PCA in the general population of men age 50 years or older (17), the PPV is 91.15%, and the NPV is 93.12%.

Reproducibility. The reproducibility of SELDI spectra, *i.e.*, mass location and intensity from array to array on a single chip (intra-assay) and between chips (interassay), was determined using the pooled normal serum quality control sample. Seven proteins in the range of 3,000–10,000 Da observed on spectra randomly selected over the course of the study were used to calculate the coefficient of variance. The intra-assay and interassay coefficient of variance for peak location was 0.05%, and the intra-assay and interassay coefficient of variance for normalized intensity (peak height or relative concentration) was 15% and 20%, respectively (data not shown). Masses that were within 0.18% mass accuracy between spectra were considered to be the same. Most important was the observation that randomly selected samples, blinded to the person performing SELDI and rerun

Table 2 *Decision tree classification of the prostate training and test sets*

Sample	Normal	BPH	PCA	Misclassified rate
A. Training set				
HM (N = 82)	78 (95.12%)	0 (0.00%)	4 (4.88%)	4 (4.88%)
BPH (N = 77)	0 (0.00%)	72 (93.51%)	5 (6.49%)	5 (6.49%)
PCA stage T_1, T_2 (N = 84)	2 (2.38%)	0 (0.00%)	82 (97.61%)	2 (2.38%)
PCA, stage T_3, T_4 (N = 83)	2 (2.40%)	0 (0.00%)	81 (97.59%)	2 (2.41%)
Total no. of samples (N = 326)				13 (3.99%)
Sample	**Normal**	**BPH**	**PCA**	**Misclassified rate**
B. Test set				
HM (N = 15)	15 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
BPH (N = 15)	0 (0.00%)	14 (93.33%)	1 (6.67%)	1 (6.67%)
PCA, stage T_1, T_2 (N = 15)	3 (6.67%)	0 (0.00%)	12 (80.00%)	3 (20.00%)
PCA, stage T_3, T_4 (N = 15)	1 (6.67%)	1 (6.67%)	13 (86.67%)	2 (13.33%)
Total no. of samples (N = 60)				6 (10.00%)

	Percent positive (no. positive/no. tested)					
Disease/nondisease	PCA/HM	PCA/BPH	PCA/(BPH/HM)	BPH/HM	BPH/T1,T2	Ti,T2/T3,T4
C. Differentiation of prostate disease from nondisease in the blinded test set						
Sensitivity	83 (25/30)	83 (25/30)	83 (25/30)	93 (14/15)	93 (14/15)	80 (12/15)
Specificity	100 (15/15)	93 (14/15)	97 (29/30)	100 (15/15)	80 (12/15)	87 (13/15)

months or even a year later, were correctly classified by the decision tree classification algorithm (Fig. 2*C*).

Discussion

The current standard screening approach for PCA is a serum test for PSA, and if the test is positive, biopsies are obtained from each lobe of the prostate. Although the PSA test has a sensitivity of >90%, its specificity is only 25%. This low specificity results in subjecting men to biopsies of the prostate as well as considerable anxiety when they do not have PCA detectable by biopsy. With the SELDI profiling classification approach, an overall sensitivity of 83%, a specificity of 97%, and a PPV of 96% were obtained in differentiating PCA from BPH and age-matched unaffected HM. Provided that this SELDI profiling classification system can be validated using a larger and more clinically diverse study set, this approach would have immediate and substantial benefit in reducing the number of unnecessary biopsies.

Our successful development of a diagnostic system that achieved a high PPV (96%) for the blinded test set is based on using a large, carefully chosen training set of randomly selected samples. All specimens were closely age matched. Serum samples from unaffected HM, identified as men with a negative DRE and PSA <4.0 ng/ml, were obtained from the general population during free prostate screening clinics. Nevertheless, selecting a cancer-free control population for studies described herein is difficult. It is unusual for a man with a normal PSA and normal DRE to undergo a prostate biopsy to be certain that the controls are truly negative. About the best that can be done is to select healthy controls that have potentially the lowest risk for PCA. For this study, 86 of the 96 HM cases had PSA values <2.5 ng/ml, which is considered a lower-risk group. The majority of the BPH patients had 4–10 ng/ml PSA and multiple negative biopsies, and the PCA patients had cancers ranging from small volume localized disease to local and distant metastatic disease and PSA values varying from 0 to >8000 ng/ml. Another important factor in the construction of a successful classification system was using an algorithm that could filter out the "noise" that is characteristic of mass spectrometry instruments, the spurious signals created by the EAM and chemical contaminates introduced in the assay, and the natural random daily fluctuations and sample-to-sample variability. This "normalization" process is critical in distinguishing peaks due to artifacts from the true peptide/protein peaks. It becomes even more important when considering that most all of the protein alterations between the cancer and noncancer cohorts are based on the overexpression or underexpression of proteins and not solely on their presence or absence. We believe that accurate and reproducible feature selection or peak "picking" algorithms with normalization functions is the most critical first step in developing a successful classification algorithm for the SELDI profiling data.

It was encouraging that the three study cohorts could be separated based on the overexpression or underexpression of nine peptide/protein masses. However, it was not surprising that multiple biomarkers would be required to effectively deal with the problem of tumor microheterogeneity that has plagued so many biomarker investigations. A previous study from our laboratory (12) is, to the best of our knowledge, the first report describing the concept of SELDI protein profiling as a potential diagnostic approach. This study observed that the selection of a combination of multiple proteins resolved by SELDI dramatically improved the detection rate of early-stage bladder cancer compared with a single marker (*i.e.*, urine cytology). Although the differential analysis in this latter study was conducted by cluster analysis and laborious manual visual inspection of all spectra, it did, however, demonstrate the power of SELDI profiling to facilitate the discovery of better cancer biomarkers. Furthermore, it clearly illustrated the need for a bioinformatics algorithm to effectively deal with the high dimensionality of the SELDI data. Based on the results of this previous study, we have explored several different bioinformatics models to mine and analyze the large amounts of data generated from these clinical proteomic studies. The models have included purely biostatistical algorithms, genetic cluster algorithms, support vector machines, and decision classification trees. All have obtained between 83–90% accuracy in separating PCA from the noncancer (BPH/HM) samples.[8] The classification tree model was selected because it is easy to interpret and the results can be clearly presented compared with "black box" classifiers such as neural networks and biostatistical algorithms, specifically with regard to the problems associated with the deconvolution steps required in identifying the protein peaks used in the classifiers. With the decision tree algorithm, the protein peaks used in the classifier are easily attainable by examination of the rules, and these rules are easily validated by examination of the SELDI processed spectra. Further proof of concept that coupling an artificial intelligent learning algorithm to analyze SELDI profiling data has potential as a diagnostic test is the recent report describing the use of a modified genetic algorithm that achieved a PPV of 94% in differentiating ovarian cancer from benign ovarian disease and healthy unaffected women (18). The discriminator pattern for classification of ovarian cancer in the study of Petricoin *et al.* (18) consisted of five protein masses of 534, 989, 2111, 2251, and 2465 Da. Although they used hydrophobic chip chemistry, which might be expected to bind some different proteins than those that would bind to the IMAC-3Cu chip used in the present study, it is interesting to note that the masses are distinctly different from those used in the prostate classification system. This suggests that the SELDI protein fingerprint profiling approach is detecting different protein patterns for each type of cancer. Studies in progress in our laboratory strongly suggest that this may be the case. We have observed that SELDI profiles of breast cancer, ovarian cancer, bladder cancer, and leukemia are different from each other and from the prostate classification profile described in this report.[9] To assure the robustness of our diagnostic system, the prostate classification algorithm is being challenged with non-PCAs and non-prostate diseases to determine that the protein profiling classification algorithm is specific for PCA. A similar scheme will be required of any disease-specific classification system.

One of the goals of this study was to identify markers in the prostate proteome that could potentially be used for early detection of cancer. Ongoing studies in our laboratory evaluating longitudinal serum samples over a 5–10-year period suggested that PCA may be suspected 5 or more years earlier than by PSA testing.[10] If validated with a larger number of patients, such studies will support the SELDI classification system as an early diagnostic test. However, to effectively apply this classification system for early detection, it will be essential to identify other biomarkers that can distinguish the aggressive cancers, *i.e.*, clinically important cancers, from nonaggressive cancers. Current evidence suggests that preoperative serum PSA <10 ng/ml is not a useful biomarker for predicting the presence, volume, grade, or rate of postoperative failure (4, 19). Thus, there is an urgent need for a better biological marker than PSA and all its molecular forms have been able to provide. A marker proportional to the volume of Gleason grade 4/5 (undifferentiated cancer) represents a critical need to more logically direct therapy tailored to tumor biology. Studies are in progress in our

[8] G. L. Wright, Jr., O. J. Semmes, P. Barlett, and C. Harris, unpublished observations.
[9] G. L. Wright, Jr., A. Vlahou, C. Laronga, J. Marks, and O. J. Semmes, unpublished observations.
[10] G. L. Wright, Jr., P. F. Schellhammer, and B-L. Adam, unpublished observations.

laboratory to evaluate SELDI serum spectra of pre- and postprostatectomy samples from patients who, after treatment, have biochemical evidence for recurrent disease in an effort to identify the biomarkers or risk factors that signal an aggressive cancer.

The successful use of the prostate classification system described herein relies entirely on the protein fingerprint pattern of the nine masses. Because these masses were found to be reproducibly reliably detected, only the mass values are required to make a correct classification or diagnosis. Knowing their identities for the purpose of differential diagnosis is not required. However, because knowing their exact identities will be essential for understanding what biological role these peptide/proteins may have in the oncogenesis of PCA, potentially leading to novel therapeutic targets, efforts are under way to purify, identify, and characterize these protein/peptide biomarkers. Furthermore, knowing their identities will be essential for producing antibodies for development of either classical or SELDI immunoassays, similar to the single and multiplex formats we described previously for the quantitation of PSA and prostate-specific membrane antigen (12, 20). The SELDI immunoassay format provides an alternate platform for quantitation of multiple biomarkers.

The high sensitivity, specificity, PPV, and NPF obtained by the serum protein profiling approach presented in this study demonstrate that SELDI protein chip mass spectrometry combined with an artificial intelligence classification algorithm can both facilitate discovery[11] of better biomarkers for prostate disease and provide an innovative clinical diagnostic platform that has the potential to improve the early detection and differential diagnosis of PCA.

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[11] L. H. Cazares, B-L. Adam, M. D. Ward, S. Nasim, P. F. Schellhammer, O. J. Semmes, and G. L. Wright, Jr. Normal, benign, preneoplastic, and malignant prostate cells have distinct protein expression profiles resolved by SELDI mass spectrometry, submitted for publication.

Normal, Benign, Preneoplastic, and Malignant Prostate Cells Have Distinct Protein Expression Profiles Resolved by Surface Enhanced Laser Desorption/Ionization Mass Spectrometry[1]

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ABSTRACT

Purpose: The objective of this study was to discover protein biomarkers that differentiate malignant from non-malignant cell populations, especially early protein alterations that signal the initiation of a developing cancer. We hypothesized that Surface Enhanced Laser Desorption/Ionization-time of flight-mass spectrometry-assisted protein profiling could detect these protein alterations.

Experimental Design: Epithelial cell populations [benign prostatic hyperplasia (BPH), prostate intraepithelial neoplasia (PIN), and prostate cancer (PCA)] were procured from nine prostatectomy specimens using laser capture microdissection. Surface Enhanced Laser Desorption/Ionization-time of flight-mass spectrometry analysis was performed on cell lysates, and the relative intensity levels of each protein or peptide in the mass spectra was calculated and compared for each cell type.

Results: Several small molecular mass peptides or proteins (3000–5000 Da) were found in greater abundance in PIN and PCA cell lysates. Another peak, with an average mass of 5666 Da, was observed to be up-regulated in 86% of the BPH cell lysates. Higher levels of this same peak were found in only 22% of the PIN lysates and none of the PCA lysates. Expression differences were also found for intracellular levels of prostate-specific antigen, which were reduced in PIN and PCA cells when compared with matched normals. Although no single protein alteration was observed in all PIN/PCA samples, combining two or more of the markers was effective in distinguishing the benign cell types (normal/BPH) from diseased cell types (PIN/PCA). Logistic regression analysis using seven differentially expressed proteins resulted in a predictive equation that correctly distinguished the diseased lysates with a sensitivity and specificity of 93.3 and 93.8%, respectively.

Conclusions: We have shown that the protein profiles from prostate cells with different disease states have discriminating differences. These differentially regulated proteins are potential markers for early detection and/or risk factors for development of prostate cancer. Studies are under way to identify these protein/peptides, with the goal of developing a diagnostic test for the early detection of prostate cancer.

INTRODUCTION

Prostate cancer is the most common cancer and, second to lung cancer, causes the greatest number of cancer deaths in American males (1). The PSA[3] serum test has contributed to earlier detection, however, 65–75% of moderately elevated PSA levels are attributed to BPH, often resulting in unnecessary biopsies (2). Several approaches have been undertaken to improve the PSA test such as measuring PSA velocity (3), PSA density (4), and assessing ratios between free, complexed, and total PSA serum values with various degrees of success (5). Combinations of markers such as free PSA, IGF-I, and IGF-binding protein 3 have resulted in improved diagnostic discrimination between BPH and prostate cancer (6). It is becoming increasingly clear that because of the inherent molecular heterogeneity and multifocal nature of prostate cancer (7), additional improvement in early detection, diagnosis, and prognosis will likely require the measurement of a panel of biomarkers.

The proteome is the full complement of proteins that regulate the physiological and pathophysiological phenotype of a cell. Because proteins initiate all cell functions and pathways, identifying differentially expressed proteins between normal and pathological states can lead to a better understanding of the

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[3] The abbreviations used are: PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; IGF, insulin-like growth factor; LCM, laser capture microdissection; MS, mass spectrometry; TOF, time of flight; SELDI, Surface Enhanced Laser Desorption/Ionization; PIN, prostate intraepithelial neoplasia, PCA, prostate cancer; IMAC, Immobilized Metal Affinity Capture; N, normal.

EXHIBIT 3

cellular mechanisms involved in disease. Some proteins are down-regulated and others are up-regulated with the onset of disease, depending on a protein's specific function, whereas others undergo disease-specific posttranslational modifications (8–10). The identification of changes in protein expression and modification that occur in the early stages of a developing cancer could lead to the discovery of protein biomarkers and novel strategies for the improvement of early detection, diagnosis, and therapy of cancer. Therefore, examining the proteome of a cell holds great potential for the discovery of new biomarkers.

As a result of the microheterogeneity of organ-based cancers, studies of pure cell populations are required to achieve precision in the search for disease-associated biomarkers. LCM microscopes have been used successfully for the procurement of pure populations of cells for genetic analysis (11, 12), protein expression changes in cancer cells using two-dimensional electrophoresis (13), and MS (14–16). Advances in MS have lead to the evolution of several proteomic applications: from the mapping of peptide digests of proteins isolated from two-dimensional electrophoresis to direct and rapid proteome profiling of cells and body fluids. For example, matrix-assisted desorption ionization-TOF-MS has been used to look for protein changes in breast cancer cell lines (17) and in the serum of cutaneous melanoma patients (18). In addition, tandem MS systems are capable of extracting peptide sequence information for sequence tagging and protein identification (19). Another innovative MS technology, SELDI, has been used to compare the patterns of protein expression in two physiological states of *Yersinia pestis* (20) and in the profiling of amyloid β peptide variants (21). Our laboratory has successfully applied SELDI to the identification of specific protein changes in the urine of bladder cancer patients (22) and the detection of prostate cancer-associated biomarkers, PSA, prostate-specific membrane antigen, prostate acid phosphatase, and prostate secretory protein in cell lysates, serum, and seminal plasma (16). Using the various affinity surfaces of Proteinchip arrays, SELDI can reduce complex protein mixtures to sets of proteins with common properties. The advantage of the SELDI protein profiling method is the ability to simultaneously detect multiple protein changes with a high degree of sensitivity (pmol to amol; Ref. 23) in a rapid high throughput process. Clear spectra are obtained with predominately singly charged ions and mass deviations of <0.02% for internally calibrated spectra (24, 25). This precision makes it possible to delineate very small proteins and peptides, as well as differential posttranslation modifications such as phosphorylation and glycosylation (26). Recently, SELDI protein profiling has been shown to provide reproducible and specific protein patterns of LCM cell lysates harvested from different cancer types and grades (15, 27).

This report describes the combinatorial use of LCM and SELDI technologies to detect protein differences in cell lysates of pure populations of normal, benign (BPH), premalignant (PIN), and malignant prostate (PCA) cells. The objectives of this study were to discover potential biomarkers that could be used to differentiate malignant from the nonmalignant cell populations, especially early protein alterations that signal the initiation of a developing cancer. The latter would be especially useful as potential markers for early detection and/or as risk factors for development of prostate cancer. Differential expression of several individual protein peaks was observed for BPH, PIN, and PCA epithelial cells with respect to the expression levels found in matched normal epithelial cells. Combinations of these signature or differentially regulated proteins/peptides could distinguish PCA and PIN from normal and BPH. However, in most cases, it was difficult to differentiate PCA from high-grade PIN. Thus, these protein alterations could represent early signals of a developing malignant lesion and may be useful as markers of early detection.

MATERIALS AND METHODS

Patient Specimens. Prostate tissues were procured from consenting patients undergoing radical prostatectomy. The age of the patients ranged from 44 to 68 years and consisted of five Caucasians and four African Americans. The tissues were processed immediately and stored in the Virginia Prostate Center's Bio-repository. Tissue pieces harvested for LCM were immediately embedded in optimal cutting temperature compound and stored at −80°C. One cryosection was H&E stained and examined by a pathologist to identify cells of interest for microdissection. Mirrored-stained sections, fixed in formalin and paraffin embedded, were also used to further aid in the identification of specific cell types. Additional serial frozen sections at 8 μm were used for microdissection.

LCM. Pure populations of normal luminal epithelia, BPH, PIN, and PCA epithelial cells were microdissected from frozen tissue sections using the PixCell II Laser Capture Microdissection Microscope (Arcturus Engineering, Inc., Mountain View, CA) essentially as described by Emmert-Buck *et al.* (28). The procedure for staining frozen sections for LCM was followed with slight modifications: the hematoxylin step was omitted and protease inhibitors (Complete; Roche Biomedical Indianapolis, IN) were added to the staining baths. A total of 1000 laser shots totaling 3000–6000 cells was procured for each cell type. Matched benign and diseased epithelial cell types were harvested from each prostate sample. In some cases, stroma cells were also procured from the same section directly adjacent to the cells of interest. Each cell population was estimated to be >98% homogeneous based on careful examination of captured cells by the pathologist. Samples were standardized by total number of laser shots, and duplicate samples were captured from the same areas of each serial section to check reproducibility.

Cell Lysates and SELDI Proteinchip Array Binding. Cell lysates were immediately prepared after microdissection by adding 4 μl of a lysis buffer containing 20 mM HEPES (pH 8.0) with 1% Triton X-100 directly on the LCM cap. Each lysate was diluted 1:10 in PBS buffer, giving a total volume of 40 μl. Lysates were vortexed for 10 min at 4°C and centrifuged briefly to remove cellular debris. The supernatant was added to an IMAC3 Proteinchip Array (Ciphergen Biosystems, Inc., Fremont, CA), pretreated with 100 mM $CuSO_4$ following the manufacturer's instructions. This surface was chosen because it produced the most robust spectra of the LCM lysates and for its ability to bind phosphorylated proteins. A bioprocessor (Ciphergen Biosystems, Inc.) was fitted on top of the chip arrays to permit the addition of the 40-μl sample. To control for variation,

cell lysates harvested from each prostate tissue were analyzed on a single biochip. The array was then incubated with the diluted lysate overnight at room temperature on an orbital shaker. After removal of the lysate, each spot was washed twice with PBS, followed by a final water rinse.

SELDI Analysis. The arrays were allowed to air dry, and a saturated solution of sinapinic acid (Ciphergen Biosystems, Inc.) in 50% (v/v) acetonitrile and 0.5% (v/v) trifluoroacetic acid was added to each spot. TOF mass spectra were generated in a Ciphergen Protein Biology System II by averaging 120 laser shots collected in the positive mode at laser settings of 225 and 280. Data were calibrated externally using purified peptide and protein standards.

Protein Profile Evaluation and Peak Expression Scoring. Spectra were analyzed with the Ciphergen Peaks 2.1 software and relative abundance for each peak were calculated as follows. The relative abundance of the proteins was subdivided into three classes: low (+), 1–30% of spectral scale; medium (++), 31–60%; and high (+++), 60–100% and analyzed for each matched set of cell types. Numerical values were then assigned to the abundance levels (*i.e.*, (+), 33; (++), 66; and (+++), 100) and averaged for each cell type to represent the protein expression between prostate cell types in graphical form.

Statistical Analysis. Sensitivity is defined as the percentage of diseased (BPH/PIN/PCA) cell types for which the biomarker of interest is present (true positive/total number of diseased lysates $\times$ 100). Specificity is defined as the percentage of cell types for which the biomarker of interest is not positive (true negative/total number of lysates without disease $\times$ 100). The statistical significance of the differences in peak expression scores between all possible pairs among the four cell types was calculated using the Wilcoxon signed rank test. A logistic regression analysis was then performed using the most significant differentially expressed proteins.

RESULTS

Sample Harvesting and SELDI Profile Evaluation. Pure populations of organ-matched benign (normal or BPH), PIN (high grade), and PCA epithelial cells were obtained from nine prostatectomy specimens examined. In four of the prostate specimens, all four cell types were identified and harvested, and PIN cells were obtained for all nine prostate tissues. The total number of cell types for each group was as follows: eight normal, seven BPH, nine PIN, and seven PCA. An average of 5000 cells was microdissected in duplicate for each cell type, resulting in 62 cell lysates analyzed by SELDI-TOF-MS. In two samples, we were able to microdissect two different foci of PIN and PCA. Additionally, adjacent stroma cells were microdissected from a selected subset of tissues to compare with the epithelial cell profiles.

Visual Analysis of SELDI Data Revealed Differential Protein Profiles. Processing the lysates on an immobilized metal affinity capture surface pretreated with $CuSO_4$ resolved between 50 and 90 protein or peptide peaks in the mass range of 3 to 100 kDa. Fig. 1 is a representative spectrum of the protein profile of a PCA cell lysate. The advantages of the SELDI technology over two-dimensional electrophoresis in resolving molecular mass protein or peptide species below *m/z* 10,000 (10 kDa) is evident. The protein profiles of each set of matched lysates were compared visually to identify differences. Expression profiling of the samples revealed several protein pattern differences, indicating up- and down-regulation or possible altered protein processing between the prostate cell types. Fig. 2*A* is the SELDI spectra and gel-view, showing a differentially expressed group of proteins between 4000–6000 Da in epithelial cells obtained from the same prostate tissue specimen. Three peaks (4030, 4358, and 4753 Da) are present or up-regulated in the PIN and PCA lysates. Fig. 2*B* is a composite SELDI gel-view of matched cell types obtained from three different prostatectomy specimens exhibiting increased expression of a peak at an average mass of 4749 Da in the PIN and PCA samples. Duplicate samples exhibited a high degree of reproducibility. In contrast, regions of heterogeneity were present in the protein profiles derived from two different foci of PCA in patient 2 and two foci of PIN in patient 3. However, overexpression of the 4749 Da protein is still observed. Profile differences were also found in the BPH cell lysates. Fig. 2*C* is an example of a peak at 5666 Da, which appears to be up-regulated in the BPH epithelial cells when compared with the spectra of the other matched cell types.

Differential Expression of Intracellular PSA Was Observed between Prostate Cell Types. To assess the potential of the SELDI technology, initial studies in our laboratory focused on the detection of known prostate cancer markers (16). Prostate-specific membrane antigen, prostate acid phosphatase, prostate secretory protein, and PSA were detected from cell lysates and body fluids. In this study, a peak of average mass 28.4 kDa was detectable in the protein profiles of epithelial cells using the IMAC surface pretreated with $CuSO_4$. This mass is consistent with the molecular mass of free PSA (29) and has been confirmed as such in our laboratory from LCM prostate cells lysates using a SELDI immunoassay (data not shown).

To determine how the expression of intracellular PSA differed between each cell type, the PSA peaks were compared in the protein profiles of organ-matched sets of lysates. Differential PSA expression between the organ-matched cell types was observed. In 5 of 9 of the PIN samples and 4 of 7 of the PCA samples, the PSA peak was reduced when compared with matched normal epithelia. An example of this decrease in intracellular PSA levels can be seen in Fig. 3. A large PSA peak at 28,393 Da was present in the normal prostate epithelia but, as expected, is absent from adjacent stroma cells. The PIN and PCA cells had a greatly reduced PSA peak. Normal epithelial cells procured directly adjacent to the PCA foci, however, exhibited a large amount of intracellular PSA. Serum PSA values for patients donating tissue for this study were between 5.5 and 26.2 ng/ml. Unfortunately, no correlation could be made between relative intracellular PSA levels in PCA cells observed in the SELDI profiles and serum PSA values.

Identification of Differentially Expressed Peaks of Diagnostic Value. A visual comparative analyses of the SELDI profiles suggested that several proteins exhibited differential regulation between the four cell types. However, to more precisely select proteins with high biomarker potential, it was necessary to standardize peak levels throughout the matched populations. The relative abundance of the peaks was subdi-

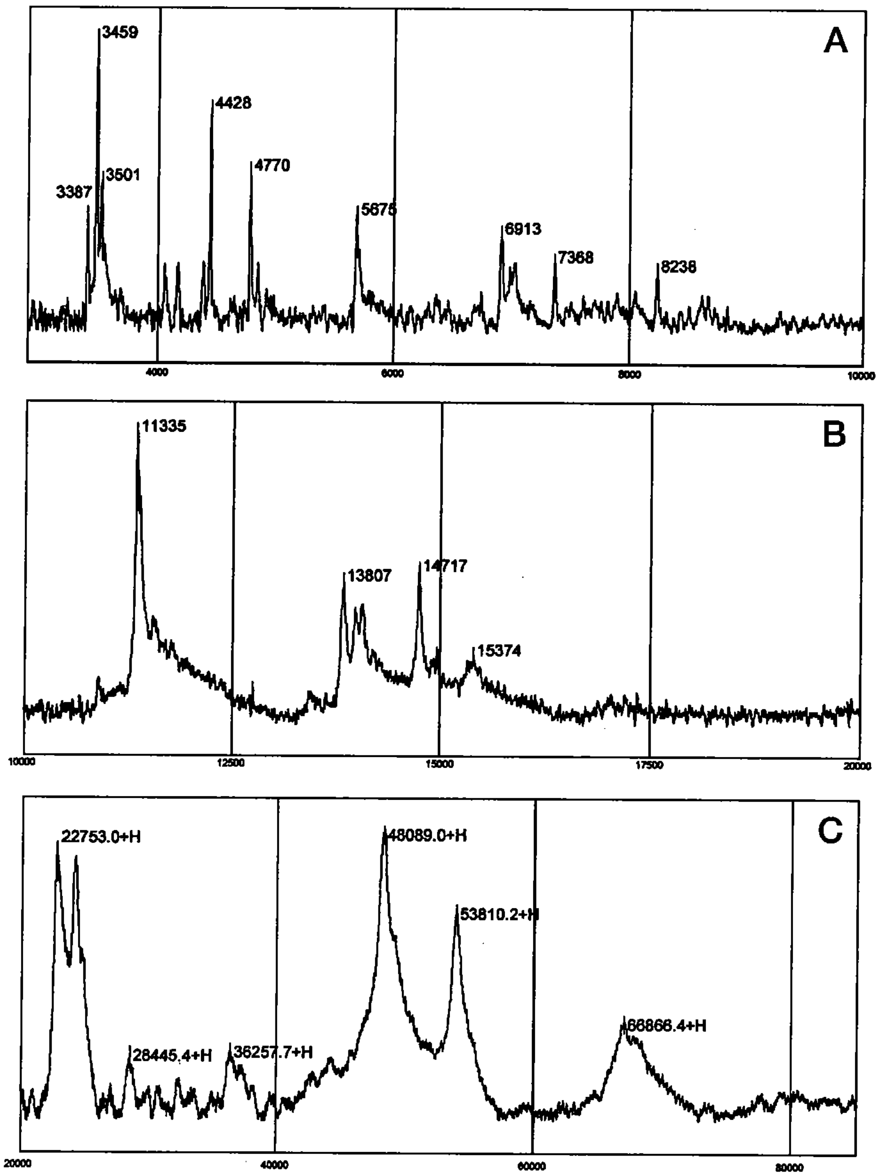

Fig. 1 Representative SELDI spectra of one LCM sample from PCA epithelial cells in *m/z* ratios of 3000–10000, 10000–20000, and 20000–80000.

vided into three classes: low (+), 1–33% of spectral scale; medium (++), 33–66%; and high (+++), 66–100%. Each individual spectrum was expanded in specific mass ranges to assist in determining the relative levels of each observed peak. An in-house computer program was used to cluster the peaks to obtain an average mass for each peak within a mass error range of 0.15%. Of an average of 70 peaks commonly observed, 14 (21%) of these peaks displayed some expression differences (up- or down-regulation or altered processing) between the epithelial cell profiles (see Table 1). Most species in this mass range were present in epithelial cell types only with the exception of peaks at 3448 Da and the 4361 Da peak, which were also present in adjacent stroma cells. The scores in Table 1 were converted to numerical values and plotted in a histogram showing the average differences in expression levels between cell types subtracted from the expression level in normal epithelial (see Fig. 4). Thus, overexpression and underexpression for each peak listed was normalized and evaluated for BPH, PIN, and PCA cells.

Proteins Overexpressed in BPH, PIN, and PCA Identified from SELDI Profiles. Generally, the PCA and PIN levels of expression profiles were similar. As seen in Fig. 4, there were higher levels of a group of small molecular mass peptides/proteins between 3000 and 5000 Da in the PIN and PCA profiles. The first of these, 3448 Da, was found at increased levels in the BPH, PIN, and PCA samples. Three peaks (4036, 4361, and 4749 Da) were overexpressed in PIN and PCA with the highest level of abundance in PCA lysates. A summary of

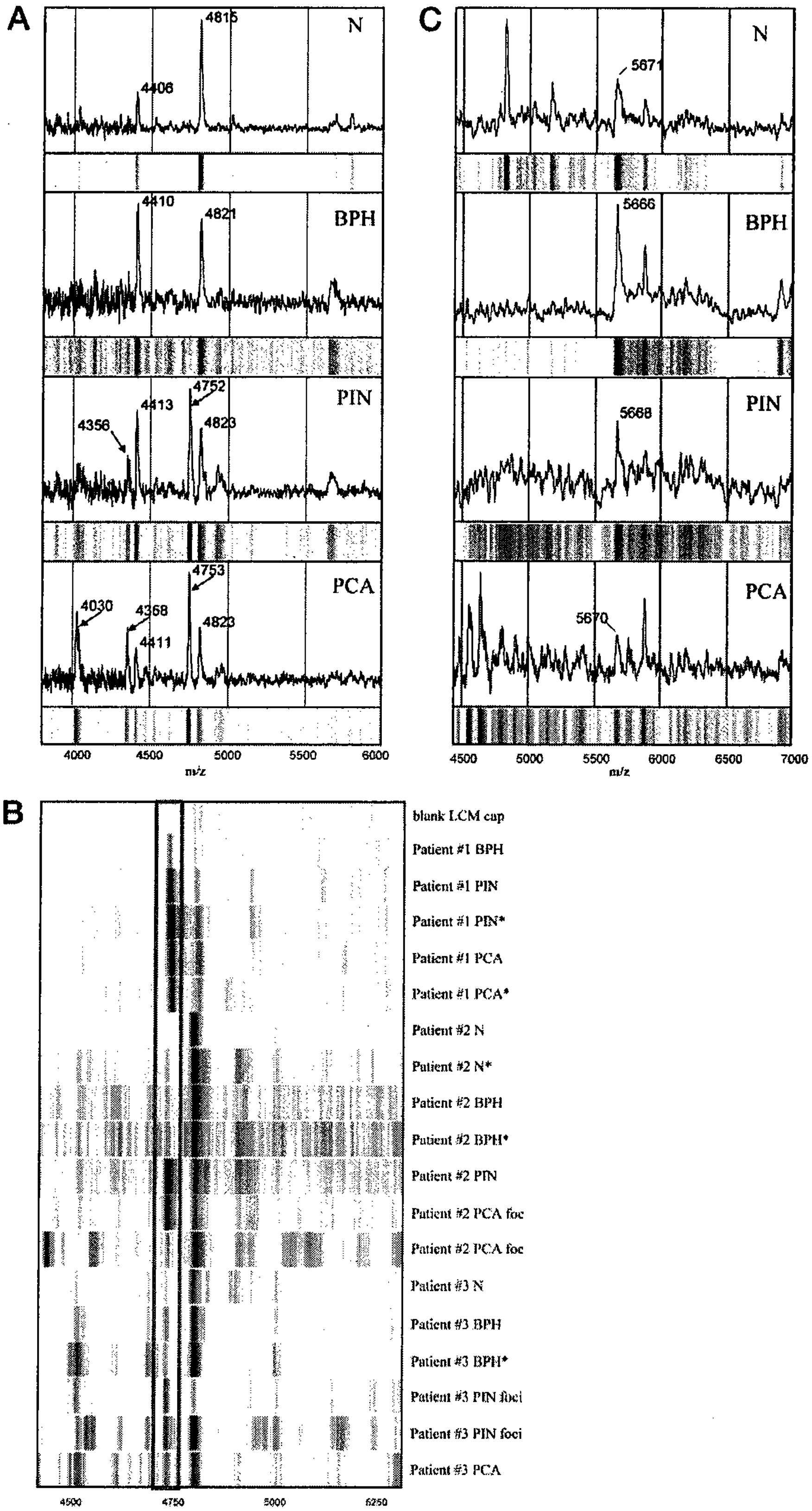

Fig. 2 *A*, representative spectra and gel-view* of matched prostate epithelial cell lysates (normal BPH, PIN, and PCA) showing protein alterations in the range of 4000–5000 Da (*m/z*). (*Arrows* indicate overexpressed peaks in PIN and PCA). *Gray scale display of the raw spectra called gel-view because it looks like a stained one-dimensional electrophoresis gel. *B*, SELDI gel-view protein profiles of lysates prepared from cells procured from different prostatectomy specimens. The *box* identifies a peak with an average mass of 4749 Da that appears to be overexpressed in PIN and PCA epithelial cells. Replicate samples(*) show good reproducibility of the protein patterns. The different protein patterns observed for two different PCA foci in patient 2 and the two PIN foci in patient 3 may be the result of genetic heterogeneity. *C*, representative spectra and gel-views of matched prostate epithelial cell lysates showing increased expression of a peak at *m/z* of 5666 in the BPH sample. N, normal.

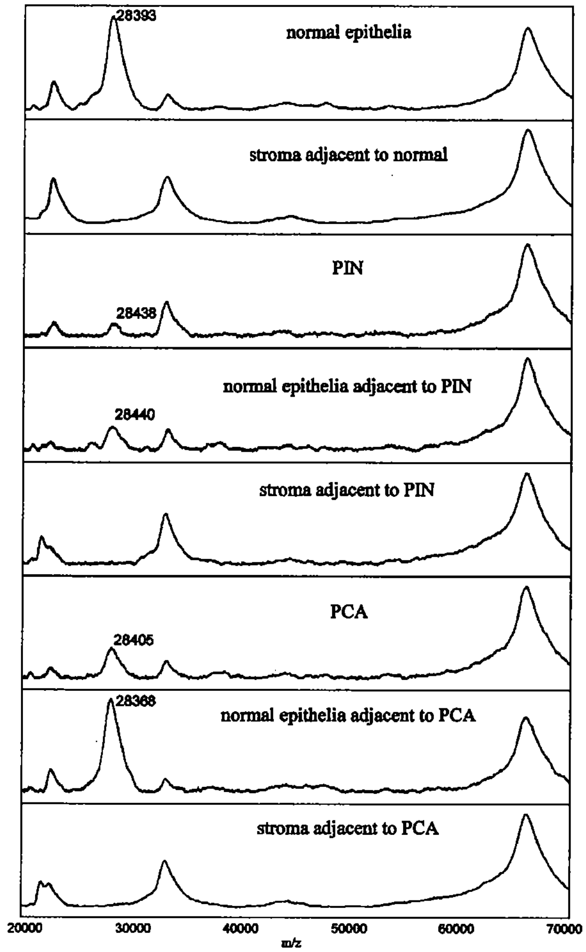

Fig. 3 Example of protein peaks found between 20,000–70,000 Da. Differential expression of a peak at 28,400 Da thought to be PSA was observed in epithelial cell lysates but absent in lysates from adjacent stromal cells. The intracellular-free PSA appears to be underexpressed in the PIN and PCA epithelial cell lysates.

the percentage of each cell type exhibiting those most significantly increased or decreased proteins is presented in Table 2. The peaks at 4036 and 4361 Da were overexpressed in 71% of the PCA lysates and 44% of the PIN samples. Additionally, two peaks (4639 and 2418 Da) were specific to PCA cell lysates but were seen in only 43% of PCA samples tested. The peak at 4749 Da was up-regulated in 67 and 57% of PIN and PCA lysates, respectively, with the highest overall peak expression value in the PCA lysates. Overexpression of a 14,696 Da peak was present in 56% of the PIN cell lysates. Also of note was a large increase in expression of a peak at 5666 Da found in 86% of the BPH profiles. Two high molecular mass proteins (48,208 and 53,836 Da) were also found to have increased expression in the BPH cells.

The significance of the expression differences between cell types was evaluated using the Wilcoxon signed rank test (Table 3). Differences were found to be significant ($P <$ 0.05) for BPH *versus* PIN and BPH *versus* PCA for overexpression of the 4036 Da peak. A comparison of N *versus* PCA approaches significance ($P = 0.059$) for this peak. Additionally, significant differences were observed for N *versus* PCA and BPH *versus* PIN in overexpression of the 4361 Da peak. Overexpression of the 4749 Da peak was significant for N *versus* PIN and approaches significance for BPH *versus* PIN. The 5666 Da protein was significantly up-regulated when comparing N *versus* BPH and, interestingly, is significantly down-regulated in BPH *versus* PIN and BPH *versus* PCA. No correlation could be made between age or race of the patients and the appearance of differentially expressed or processed peaks.

Proteins Underexpressed in BPH, PIN, and PCA Profiles. As seen in Fig. 4, a few proteins were underexpressed in BPH, PIN, and PCA cells when compared with the expression levels in normal epithelial cells. Expression of the 5666-Da peak, which was overexpressed in BPH profiles, was reduced in 22% of PIN and 71% of PCA cell lysates at significant levels (see Tables 2 and 3). In addition, decreased expression of an 11,744-Da protein and PSA (28,442 Da) was found in the BPH, PIN, and PCA cell lysates. The reduced expression of intracellular PSA was found in 56 and 57% of PIN and PCA cell lysates, respectively. Furthermore, this underexpression of PSA was significant in N *versus* PIN and approaches significance ($P = 0.066$) for N *versus* PCA (Table 3).

Biomarker Combination of Identified Peaks Improved Prediction of Cell Lysate Disease State. Because no single peptide or protein was discovered to be differentially expressed in all of the PIN or PCA profiles, various combinations of selected proteins were evaluated to identify a panel of markers (expression levels) that could improve disease classification of each specific cell type (Table 4). A biomarker combination was classified as positive if any marker in the combination was present in a sample and negative if none of the markers were detected in a specimen. Because most of these markers were overexpressed in both PCA and PIN, the combinations did not improve the specificity for each of the diseased cell types when evaluated individually.

Better discrimination was achieved by combining the benign cell types (normal, BPH) and comparing them to diseased cell types (PIN or PCA). The sensitivity and specificity of each marker for benign *versus* disease were then calculated both individually and in additive combinations. By combining markers 1 (p4036) and 3 (p4639), there was an improvement in the sensitivity for PCA from 71 to 86% while maintaining a specificity of 100% for PIN or PCA combined. This combination of markers also identified 44% of the PIN samples. Combining marker 2 (p4361) and marker 3 (p4639) increased the sensitivity for PCA to 100% while the specificity decreased to 87% for PIN and PCA. By combining markers 2 and 4 (p4749), 100% of the PCA and 89% of the PIN samples could be correctly identified with a slight decrease in specificity. Combinations involving three or more of the markers did not improve the overall specificity and sensitivity. The 5666-Da protein (marker 5, Table 4) had a sensitivity of 86% and specificity of 88% for detecting BPH. This marker also provided some discrimination between BPH and PIN lysates having 22% sensitivity and 68% specificity for PIN epithelia.

Table 1 Comparison of the expression levels[a] of proteins in lysates of LCM-procured prostate cells (epithelial and stromal)

Molecular mass (Da)	3,448	4,036	4,361	4,413	4,639	4,749	5,666	8,445	11,744	14,696	24,184	28,422	48,308	53,830
Speciman no.														
CA3491														
BPH	+	0	0	0	0	+	+++	0	0	+	0	+	++	+
PIN	++	+	0	++	0	++	0	0	0	+++	0	++	+	0
PCA	+++	+	0	++	0	+++	++	0	+	++	+++	+	+++	++
CA3495														
N[b]	++	+	+	+	0	0	+	0	+	+++	0	++	+	+
BPH	+++	0	0	++	0	0	++	0	+	++	0	+	++	++
PIN	+++	+	+	++	0	++	+	0	++	++	0	++	+++	+
PCA	+++	++	++	+	0	+++	0	0	+	++	+++	+	+	+
CA3501														
N	+++	0	++	+	0	0	0	0	++	+	0	++	++	+
BPH	+++	0	++	+	0	+	0	0	++	++	0	+	0	0
PIN	+++	+	++	+	0	+	0	0	++	++	0	++	++	+
CA3510														
N	+++	0	0	0	0	0	0	+	++	0	0	+++	0	0
PIN	+++	0	0	0	0	+	0	0	0	+++	0	++	0	0
PCA	+++	0	0	0	0	+	0	0	0	0	+	+++	0	0
CA3517														
N	++	0	+	+	0	0	+	0	+++	0	0	+++	+	0
BPH	++	0	++	++	0	0	++	0	+	0	0	+++	++	+
PIN	+	0	++	+	0	+	+	0	0	0	0	0	0	0
PCA	+++	+	++	+	+	0	0	0	0	0	0	0	0	0
CA3511														
N	+	0	0	0	0	0	+	0	+	0	0	++	++	+
BPH	++	0	+	+	0	0	+++	0	0	0	0	++	+	+
PIN	++	+	++	+	0	+	+	0	0	+	0	++	+	+
PCA	+	+++	++	+	+	+	+	+	+	0	0	++	+	+
CA3535														
N	0	0	0	0	0	0	+++	0	+	0	0	+++	+	+
PIN	++	0	0	0	0	0	+	++	+	0	0	+	0	0
PCA	++	0	+	0	+	0	0	+	+	0	0	+	0	0
CA3539														
N	0	0	+	0	0	0	0	0	+	0	0	+	0	0
BPH	++	0	+	0	0	0	++	0	+	0	0	0	+	+
PIN	+++	0	++	0	0	0	+	0	+	0	0	0	0	0
CA3546														
N	+	0	0	0	0	0	+	0	+	0	0	++	+	+
BPH	++	0	0	0	0	0	+++	0	+	0	0	++	+	+
PIN	++	+	+	0	0	0	++	+	0	+	0	0	0	0
PCA	+++	+	++	0	0	0	0	0	+	+	0	0	0	0
Adjacent stroma cells														
To N/BPH	+	0	+	0	0	0	0	+	0	0	0	0	0	0
To PIN/PCA	++	0	++	0	0	0	0	+	0	0	0	0	0	0

[a] Relative expression levels of proteins: +, 1–33% of SELDI spectral scale; ++, 33–66%; and +++, 66–100%, 0, peak not detected.
[b] N, normal.

Multivariable analysis of the peak expression data was evaluated to determine whether the simultaneous overexpression or underexpression of several proteins in combination could be used to predict benign *versus* diseased cell lysates. Logistic regression analysis was performed with the seven most significant differentially expressed peaks [4,036, 4,361, 4,413, 4,639, 4,729, 5,666, and 28,422 Da (PSA)]. As seen in Table 5, a predictive equation based on diseased (PIN/PCA) *versus* benign (normal/BPH) cell lysates resulted in 93.3% specificity and 93.8% sensitivity for PIN or PCA cell lysates. Therefore, these biomarkers in combination may have clinical value, especially if detectable in biopsy or body fluid samples

DISCUSSION

The development of cancer is a multistep process encompassing multiple events involving oncogenic and tumor suppressor gene products. These events can occur pre- or posttranslationally and will be reflected in differential changes in a myriad of proteins. Analyzing and interpreting the proteomic changes that occur in prostate disease progression is a daunting task made even more difficult by the biological heterogeneity of the disease. Advances in TOF-MS, resulting in the SELDI technology, have provided an approach for the sensitive and direct analysis of proteins in complex biological samples. Previous studies in this and other laboratories have demonstrated the successful application of this technology to profile a few microdissected prostate cell specimens (15, 16, 30). In this study, we combined LCM with SELDI to generate protein profiles from 62 prostate cell lysates derived from nine prostatectomy specimens. Our results demonstrate that the protein profiles of normal prostate epithelia, BPH, PIN, and PCA display discriminating differences.

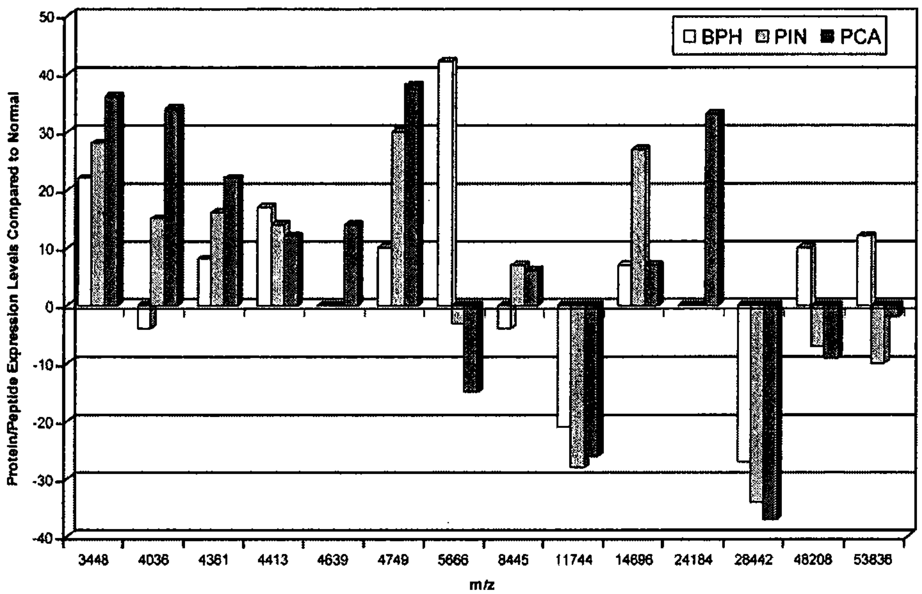

Fig. 4 Expression levels of peptide/proteins compared with the expression levels in matched normal epithelial cells. The average expression in normal cell lysates was subtracted from the average expression levels found in the other cell types. *Bars above 0,* overexpression; *bars below 0,* underexpression.

Table 2 Percentage of samples (BPH/PIN/PCA) displaying differential expression of selected proteins when compared with matched normal samples

	BPH % of samples		PIN % of samples		PCA % of samples	
Molecular mass (Da)	Overexpression	Underexpression	Overexpression	Underexpression	Overexpression	Underexpression
3,448	57	0	67	11	71	0
4,036	0	0	44	0	71	0
4,361	29	14	44	0	71	0
4,413	43	0	33	0	29	14
4,639	0	0	0	0	43	0
4,749	14	0	67	0	57	0
5,666	86	0	22	22	0	71
8,445	0	0	22	11	29	0
11,744	0	43	11	44	14	29
14,696	14	14	56	0	29	14
24,184	0	0	0	0	43	0
28,422	0	43	11	56	0	57
48,308	57	43	11	56	14	43
53,830	29	14	0	33	14	14

Protein extracts prepared from prostate cell types were analyzed using an IMAC3 protein biochip pretreated with $CuSO_4$. On average, 70 protein peaks were detected from the cell lysates using this surface. Overall, there were a relatively large number of common peaks in the benign and diseased epithelial cell profiles and very few peaks that would, based on presence or absence, be candidate biomarkers for the disease progression and/or diagnosis of prostate cancer. It was therefore determined that calculating expression levels of peaks would enhance the significance of the results and identify possible expression differences between the samples. Peak abundance levels were calculated for diseased cell profiles as compared with levels found in matched benign cell types. Of the common 70 peaks observed, 15 (21%) of these peaks displayed dysregulation in the diseased profiles.

Several small molecular mass peptides or proteins (3000–5000 Da) had increased expression levels in the PIN and PCA cell extracts. Although, the clusters of peaks in this range could originate from proteolysis and cleavage products of larger proteins, they nonetheless were consistently detected in common cell types and were considered part of the general profile. The chymotrypsin-like activity of PSA has been shown to facilitate the proteolysis of semenogelin from seminal plasma (31), and IGF-binding protein 3 (32). Such stable cleavage products of proteins may be indicative of changes occurring in the prostate disease cycle. Furthermore, because the IMAC surface can bind phosphorylated peptides or proteins, it is feasible that some of the changes observed are attributable to differential phosphorylation of the proteins in the diseased cell types. Of interest in this study, two peaks were identified with an average mass of 4827

Table 3 Wilcoxon signed ranks test of peak expression differences

Peak (Da)	N[a] *vs.* BPH	N *vs.* PIN	N *vs.* PCA	BPH *vs.* PIN	BPH *vs.* PCA	PIN *vs.* PCA
4,036	0.317	0.083	0.059	0.025[b]	0.039[b]	0.102
4,361	0.564	0.059	0.038[b]	0.046[b]	0.102	0.083
4,413	0.083	0.157	0.317	0.655	1.000	0.317
4,639	1.000	1.000	0.083	1.000	0.157	0.083
4,749	0.317	0.034[b]	0.102	0.059	0.109	0.564
5,666	0.038[b]	1.000	0.059	0.024[c]	0.039[c]	0.408
28,422 (PSA)	0.083	0.041[c]	0.066	0.680	0.180	0.564

[a] N, normal.
[b] Significant *P*s ($\leq$0.05) based on overexpression as compared with N or BPH.
[c] Significant *P*s ($\leq$0.05) based on underexpression as compared with N or BPH.

Table 4 Sensitivity and specificity of single and combinations of markers for the detection of diseased cells

	Sensitivity		Specificity		Sensitivity/Specificity
	PCA	PIN	PCA	PIN	PIN or PCA
(Marker) expression[a]					
(1) increase of *m/z* 4036	71%	44%	83%	77%	56%/100%
(2) increase of *m/z* of 4361	71%	44%	75%	68%	56%/87%
(3) presence of *m/z* 4639	43%	0%	100%	N/A	19%/100%
(4) increase of *m/z* of 4749	57%	67%	71%	71%	63%/94%
Combination					
1 + 2	86%	67%	67%	64%	75%/87%
1 + 3	86%	44%	83%	77%	63%/100%
2 + 3	100%	56%	75%	68%	56%/87%
1 + 4	86%	78%	67%	68%	81%/94%
2 + 4	100%	89%	54%	55%	94%/80%
3 + 4	86%	67%	71%	68%	75%/94%
1 + 2 + 3	86%	67%	67%	64%	81%/94%
1 + 2 + 4	100%	89%	54%	59%	94%/80%
	Sensitivity		Specificity		
	BPH	PIN	BPH	PIN	
(Marker) expression					
(5)[b] increase of *m/z* 5666	86%	22%	88%	68%	

[a] The increase in abundance of each marker was determined from matched normal or BPH epithelial cell lysates.
[b] The sensitivity and specificity of a marker found in abundance in BPH samples (5) when compared with PIN and PCA samples.

Table 5 Classification table based on logistic regression analysis using expression levels of seven proteins: [p4036, p4361, p4413, p4639, p4749, p5666, and p28422 (PSA)]

	Observed		Predicted (PIN/PCA)		
			N[a]	Y[a]	% correct
PIN/PCA	N	15	14	1	93.3 (specificity)
	Y	16	1	15	93.8 (sensitivity)
					93.5 (overall)

[a] N, no; Y, yes.

Da $\pm$ 26.5 (common in the benign cell types) and 4749 Da $\pm$ 26.1 (higher abundance in PIN/PCA; Fig. 2*A*). These two peaks may represent a dephosphorylation event occurring in transition from benign to PIN/PCA. The average mass shift between these proteins (78 Da) is close to the calculated mass shift of 79 Da for a phosphorylation event. Prominent examples of aberrant phosphorylation of proteins found in cancer studies include extracellular signal-regulated kinase 1/2 in breast cancer (33) and androgen receptor in prostate cancer (34).

It is also quite possible that these small molecules could be intact functional proteins or peptides, examples of which include prohormones, growth factors, amidated peptides, and defensins. In a recent study by Rocchi *et al.* (35), PC-3 and Du145 prostate cancer cell lines were found to produce and secrete a multifunctional amidated peptide (adrenomedullin, molecular mass ~6 kDa). In the same study, increased levels of adrenomedullin immunostaining were found in PCA epithelia when compared with normal epithelia. The activity of the enzyme peptidylglycine α-amidating monooxygenase was also demonstrated in prostate cancer cell lines. This enzyme produces α-amidated bioactive peptides from their inactive glycine-extended precursors. The importance of the role of these small molecular mass proteins may have previously been overlooked as a result of the difficulty in detection using two-dimensional analysis.

Previous studies have shown a cytogenetic link between high-grade PIN and prostate cancer strengthening its role as a precursor lesion (36). In addition, >50% of patients with high-grade PIN present with cancer detected in a subsequent biopsy (37). Therefore, the identification of proteins specifically associated with PIN would have tremendous impact as markers for the early detection of prostate cancer. In our study, PIN and

PCA cell lysates exhibited similar protein profiles underscoring the phenotypic similarity of these two disease states. Three peaks at 4036, 4361, and 4749 Da showed increased abundance in PIN and PCA lesions when compared with matched benign cell profiles. Two other peaks at 11,744 and 28,442 Da (free PSA) were decreased in BPH, PIN, and PCA cell extracts, and one marker at 14,696 Da was overexpressed in 56% of the PIN samples and only 29% of matched PCA samples. Interestingly, a few of these peaks, based on closely matched molecular masses, were found to be present in serum and seminal plasma from two of the patients donating tissue for this study (data not shown). Because these body fluid profiles were generated using the IMAC surface pretreated with $CuSO_4$, they may represent the same proteins. However, additional samples will need to be examined to confirm this result. If the markers discovered in the tumor cell lysates can be detected in serum or seminal plasma, they may aid in the early detection/diagnosis of prostate cancer. The identification of these peptides or proteins and their use as possible markers of early detection is currently under investigation in our laboratory.

In this study, most of the markers found in the PCA profiles were also present in the PIN profiles, and thus, the ability to discriminate between these two cell types was difficult. However, better discrimination could be achieved between the benign cell types (normal, BPH) and the diseased cell types (PIN or PCA combined). Because it is well established that multiple foci of PIN and PCA arise independently within the same prostate and prominent genetic heterogeneity is a common feature of prostate disease, a panel of biomarkers is the most likely solution to improvements in early detection and diagnosis. In maximizing the use of our approach, we explored a combination of biomarkers with the most significant differential expression. Combining markers 4361 and 4749 Da improved the sensitivity to 100% for the detection of PIN and PCA while maintaining 87% specificity. When we incorporated the seven most differentially expressed proteins in a logistic regression analysis, a predictive equation resulted in 93.3% sensitivity and 93.8% specificity for PIN or PCA. One of the seven peaks (4,639 Da) and an additional peak (24,184 Da) were found only in PCA cell lysates. Each of these markers was expressed in 43% of tumor samples profiled. Because the majority of our tissue samples were moderately differentiated cancer (combined Gleason scores of 6 or 7), no correlation could be made to Gleason grade of tumor with regard to the expression of the peaks we found to be differentially expressed in PCA. Future studies involving the protein profiles of poorly differentiated and metastatic prostate cancer samples would determine whether any of the selected biomarkers represent markers of metastatic potential. Identifying if a patient has a clinically significant or insignificant cancer could feasibly be determined by an antibody array analysis of the patient's biopsy (*i.e.*, lysate) using antibodies to selected biomarkers.

Differences were observed in the expression of a 28,400-Da peak, which is consistent with intracellular-free PSA. The molecular mass identified in our study closely matches the observed molecular mass of free PSA (28,430 Da), determined using ion spray MS (29). Likewise, this peak was absent from matched adjacent stroma cell lysates, indicating its specific expression in epithelial cells. Furthermore, data obtained from our immunoassay studies, also performed using the SELDI platform (16), have identified this peak from LCM cell lysates as PSA based on immunoaffinity (data not shown). In this study, normal epithelia of the prostate expressed large amounts of PSA, whereas the diseased cell types (PIN and PCA) had reduced expression levels. Normal epithelia microdissected directly adjacent to the tumor foci also expressed high levels of PSA. This decrease in intracellular PSA in PCA cells is in agreement with other studies. For example, Jung *et al.* (38) found tissue PSA levels lower in cancerous than in normal tissue from the same prostate gland, and a study by Weir *et al.* (39) found immunohistochemical staining intensity of PSA inversely correlated with histological grade of tumor. Furthermore, significant inverse correlations have been found between tissue PSA expression levels and serum PSA values (40). Interestingly, a recent report by Pawletz *et al.* (15) also found a 28-kDa protein peak (not identified as PSA) via SELDI to be down-regulated in microdissected PCA cells when compared with matched normal epithelia. Our results are consistent with the hypothesis that the increase in serum PSA in men with prostate cancer is not because of increased production of PSA by the tumor cells but rather an increased leakage of PSA from the tumor tissue into the circulation as a result of a breakdown of tissue architecture.

The BPH protein profiles also displayed some notable differences. There was an increase in abundance of peaks at 3448, 4413, and 5666 Da in the BPH lesions. Of special interest was the 5666-Da peak, found to be overexpressed in 86% of the BPH cell lysates with a specificity of 88%. Only 22% of the PIN lesions and none of the PCA lesions overexpressed this marker. Efforts are under way to characterize this protein. A biomarker indicative of BPH alone may, if secreted into serum or seminal plasma, be useful in the reduction of biopsies in patients with elevated PSA.

In conclusion, differential SELDI protein profiles were observed for cell lysates prepared from microdissected normal, BPH, PIN, and PCA epithelial cells. Several small molecular mass species were found to be overexpressed in PIN, and because they were also overexpressed in PCA, these proteins may represent early signals or signatures of a developing cancer. Additionally, one marker at 5666 Da was found to be increased in BPH and may have the ability to distinguish BPH from PCA. A combination of markers was effective in distinguishing normal/BPH from PIN/PCA with a sensitivity and specificity of 93%. Additional studies are under way to identify and characterize these potential peptide/protein biomarkers using liquid chromatography tandem MS. Once identified, characterization of their function and biological role in prostate oncogenesis may lead to their potential use as diagnostic and prognostic biomarkers as well as conceivable therapeutic targets.

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